RESEARCH

Genetic Mapping of Persistence in Tetraploid Alfalfa

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ABSTRACT

Persistence is a critical trait for alfalfa (*Medicago sativa* L.), yet the genetics of this trait are poorly understood. Herein, we characterize an F_1 alfalfa population derived from the cross between the two cultivated alfalfa subpecies for persistence in three production seasons at Ames and Nashua, IA, and one production season at Ithaca, NY. Quantitative trait loci (QTLs) underlying persistence were mapped using this population utilizing single-marker analysis and interval mapping procedures. Both parental genomes (*Medicago sativa* subsp. *falcata* and *M. sativa* subsp. *sativa*) contributed marker alleles associated with persistence, suggesting that alleles from both subspecies have potential for marker-assisted selection. Although, linkage groups 1, 2, and 7 contained putative persistence QTLs, genotype × environment interaction and location-specific QTLs suggest location-specific genetic mechanisms for alfalfa persistence. Nevertheless, in some instances, the same QTLs were identified in different years at the same location. Quantitative trait loci on linkage groups (LGs) 1 and 2 were location-specific for the Ithaca and Ames locations, respectively. The majority of alleles on LG 7 associated with persistence also exhibited association with biomass production and suggest common genetic determinants for both traits. Of particular interest was the identification of simple sequence repeat allele al37288-1a1, which associated positively with persistence in each environment of the study.

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Abbreviations: LG, linkage group; MAS, marker-assisted selection; QTL, quantitative trait locus.

Elite alfalfa (*Medicago sativa* L.) cultivars not only must have high forage yields but also must maintain their productivity and stands over several years (persistence). Persistence is a complex trait that is affected by a number of factors, including genotype, abiotic and biotic environmental factors, management, and their interactions (Riday and Brummer, 2006). Improved disease resistance and winter hardiness have been specifically identified as traits crucial to alfalfa persistence (Volenec et al., 2002). Recent findings suggested that alfalfa forage yield gains were primarily the result of improved disease resistance, probably due to improved stand longevity (Lamb et al., 2006). Heterosis for yield (Riday and Brummer, 2002a, 2005) and various agronomic traits (Riday and Brummer, 2002b) is characteristic of crosses between alfalfa subspecies [*M. sativa* subsp. *sativa* (sativa) and *M. sativa* subsp. *falcata* (falcata)] and points to the potential utility of semihybrid alfalfa cultivars (Brummer, 1999) to overcome deficiencies in current alfalfa cultivar selection programs. However, persistence of the falcata × sativa hybrids is not necessarily superior to intraspecific sativa hybrids (Riday and Brummer, 2006).

Gain in alfalfa persistence will require selection for persistence per se with more emphasis on long-term nurseries and family

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selection methods. The long-term nature of selection for persistence slows the cycle time in a breeding program, thereby limiting genetic gain. A potential mechanism for improving alfalfa persistence more quickly is the use of marker-assisted selection (MAS). Although the genetic determinants of alfalfa persistence are unknown, several agronomic-associated quantitative traits have been mapped in alfalfa, including winter hardiness (Brouwer et al., 2000), aluminum tolerance (Sledge et al., 2002), biomass production (Robins et al., 2007a,b), and forage height and regrowth (Robins et al., 2007a). Thus, quantitative trait locus (QTL) mapping of persistence may allow the identification of the genomic regions underlying alfalfa persistence.

A study was designed to characterize persistence at three disparate geographic locations in a tetraploid alfalfa population derived from a falcata × sativa cross, and to identify genomic regions underlying alfalfa persistence using a molecular genetic map and marker data created for this population (Robins et al., 2007b).

MATERIALS AND METHODS

Plant Materials

Two hundred genotypes from a WISFAL-6 (Bingham, 1993) × ABI408 (Forage Genetics International, Nampa, ID) cross were grown in the Iowa State University greenhouses at Ames, IA during the winter 1998–1999. These genotypes are the same used in previous field and laboratory studies (Brummer et al., 2000; Robins et al., 2007a,b). Each parental and F_1 genotype was clonally propagated using stem cuttings to establish field experiments.

Experimental Design

Field experiments were the same as presented previously (Robins et al., 2007a,b). Briefly, seedlings were transplanted at the Agronomy and Agricultural Engineering Research Farm west of Ames, IA, on 19 May 1998; at the Northeast Research Farm south of Nashua, IA, on 22 May 1998; and at the Cornell Agricultural Experiment Station in Ithaca, NY, on 7 to 9 June 1999. The plot design at Ames and Nashua was a quadruple, α-lattice consisting of 840 total plots; at Ithaca, it was a randomized complete block design consisting of four blocks and 824 total plots. New York had fewer plots than Iowa because of the loss of six genotypes before transplanting and the inclusion of two additional check cultivars in New York. Plots in Iowa consisted of five clones of each genotype; in New York, they consisted of seven clones, but data were only collected from the inner five clones. In Iowa, spacings were 30 cm between plants within a plot, 60 cm between plots in the same row, and 75 cm between rows. In New York, spacings were 25 cm between plants within a plot, 60 cm between plots within the same row, and 90 cm between rows.

Data Collection

The number of surviving plants in each plot were counted (plants per plot) initially in September 1998 at Ames and Nashua and in May 2000 at Ithaca. Subsequent plant counts occurred during May or June of each year through 2001. For each year, the percentage of surviving plants based on the initial counts was calculated and used as the measure of persistence.

Phenotypic Data Analysis

An initial linear model was developed to describe the observed mean value of each genotype across the two Iowa locations. This model included the effects of genotype, location, year, complete blocks nested within location, and incomplete blocks nested within complete blocks and location along with appropriate interactions. This model also accounted for the repeated nature using a split-plot-in-time or compound symmetry model. However, because genotype × environment interactions were present and because we wanted to examine marker-trait associations from year to year and from location to location, the Iowa data were reanalyzed within individual location and year combinations. The Ithaca data were analyzed separately with the same model (without the incomplete blocks nested within complete blocks). The MIXED and CORR procedures of the SAS statistical software package (Littell et al., 1996; SAS Institute, 2006) was used for the analysis. Broad-sense heritability and genotypic correlation estimates were calculated using the approach of Holland et al. (2003) and Holland (2006), respectively. Data were collected for 3 yr after initial plant counts in Iowa and 1 yr in New York. Each location-year combination is referred to as an environment. We denote the environments by a letter (A = Ames, N = Nashua, and I = Ithaca) and a number indicating the year in which persistence was measured (e.g., A1 = Ames persistence after 1 yr).

Mapping and Markers

With the exception of updating the linkage maps using the updated version of TetraploidMap software (Hackett et al., 2007) rather than JoinMap (Van Ooijen and Voorrips, 2001), all mapping procedures were described in Robins et al. (2007b). Restriction fragment length polymorphism and simple sequence repeat marker alleles are identified by the marker name (see Robins et al. [2007b] for probe and primer sources) followed by a letter indicating the parental genome that contributed the allele ("a" from WISFAL-6, "b" from ABI408, or "c" from both parents) and a distinct number for each individual allele. The linkage maps from TetraploidMap resulted in somewhat different marker order compared with the original maps created with JoinMap. Differences resulted from a different ordering algorithm used in the TetraploidMap software compared to original ordering done with JoinMap and the exclusion of markers segregating at a ratio higher than 5:1 because of the inability of TetraploidMap to place these alleles correctly. We felt the result was justified because of the ability to perform interval mapping with maps developed using the TetraploidMap software. We were limited to single marker analysis with the JoinMap-created maps.

QTL Analysis

Marker-phenotype associations, using the marker data described previously (Robins et al., 2007b), were identified using single-factor analysis of variance and the GLM procedure of SAS (SAS

Institute, 2006) as an exploratory approach. For each marker allele, the mean value of genotypes containing the allele was contrasted with those of individuals without the allele. The analysis was completed for each environment of the study. Alleles were declared to be significantly associated with a trait at $p \leq 0.01$. To identify single-marker associations with stronger statistical support, a nonparametric permutation test (Churchill and Doerge 1994) based on the family-wise error rate of Westfall and Young (1993) was also used. Associations identified via the single-marker analysis were then further characterized using the tetraploid interval mapping procedure of TetraploidMap software. Quantitative trait loci were identified based on LOD values larger than the 5% cutoff value determined through 1000 permutations.

RESULTS AND DISCUSSION

Phenotypic Characterization of Persistence

Parental and F_1 population mean persistence values were consistently high (>80%) in each environment, except N3 when persistence values decreased substantially (Table 1). Although no obvious biotic stresses were present at any of the locations, the differential effects of the environmental stresses (e.g., N3 results and the significance of genotype by environment effects in the overall model) were characteristic of difficulties previously noted in the improvement of alfalfa persistence (Volenec et al., 2002; Riday and Brummer, 2006). No differences either between parental means or between parental means and the F_1 population mean occurred in any environment (Table 1).

Persistence was highly variable among the genotypes constituting the F_1 population with values ranging between 0 and 100% depending on the environment (Table 1). Broad-sense heritability values (H^2) estimated on genotype means ranged from 0.32 in A1 to 0.74 in N2 (Table 1), indicating the importance of the genetic component underlying the trait at each location. None of the F_1 genotypes exhibited better persistence than the parents, but trangressive segregants were identified with less persistence than the parents in each environment (Table 1). Transgressive segregation was consistently associated with the same genotypes across years at a location (obviously, persistence can only decline over time) and to a lesser extent, across locations (data not shown). Phenotypic and Spearman rank correlations among values across locations within years were all low (ranging from 0.07 to 0.34), as were correlations among blocks within the same environment (all below 0.4). This population had both high and low transgressive segregants for biomass production, forage height, and regrowth (Robins et al., 2007a,b), but because the parents were quite persistent, higher persistence values were not possible.

Marker-Phenotype Associations

Sixteen alleles were associated with alfalfa persistence in at least one of the environments included in this study based on single-marker analysis of variance (Table 2). The effect of the alleles ranged from −8 to 13% (Table 2). Thirteen of the 16 alleles exhibited associations with persistence in more than one environment, but only four of the alleles (al372288-1a1, al373004a1, bg645450b1, and uga772b1) exhibited associations in more than one location, and only one allele (al372288-1a1) exhibited associations in all environments. Alleles al373004a1 (LG 7) in five environments and ms29b2 (LG 1) in I1 were significant at a family-wise error rate of 3 to 10%, depending on the environment.

Allelic associations with persistence were confined to four of the eight consensus LGs (LGs 1, 2, 3, and 7; Table 2). Alleles associated with persistence on LG 7 were also associated with other traits, including biomass production, forage height, and regrowth (Table 2; Robins et al., 2007a,b). From our findings and the results of other studies (Julier et al., 2007), this region appears to be a key area of the *Medicago* genome for productivity. Despite the common allelic associations, genotypic correlations between biomass production (Robins et al., 2007b) and persistence in the same environment were low, with only the correlation in A3 being greater than 0.3 (data not shown). Alleles on LGs 1 and 3 only exhibited association with persistence at Ithaca, while alleles on LG 2 only exhibited association at Ames. Alleles on LG 7 showed extensive associations at Ames and Nashua but had only one association at Ithaca. These results indicate that allelic effects vary among environments and suggest different genetic mechanisms of alfalfa persistence at each of these locations.

Each parent (ABI408 and WISFAL-6) contributed both positive and negative alleles for persistence (Table 2). All of the allelic associations on LGs 1 and 3

Table 1. Mean persistence values for the parents (ABI408 and WISFAL-6) and F_1 alfalfa population measured across 3 yr at Ames and Nashua, IA, and 1 yr at Ithaca, NY.

	Persistence						
	A1†	A2	A3	N1	N2	N3	I1
	%						
H^2‡	0.62 ± 0.03	0.66 ± 0.03	0.32 ± 0.05	0.71 ± 0.03	0.74 ± 0.03	0.54 ± 0.05	0.35 ± 0.05
ABI408	100	100	85	94	95	73	100
WISFAL-6	100	100	100	80	80	54	88
F_1 population mean	89	88	83	89	87	63	95
F_1 population range	0–100	0–100	0–100	0–100	0–100	0–78	25–100
$LSD_{0.05}$	28	29	33	23	25	25	23

†Experimental location and year: A = Ames, IA; N = Nashua, IA; I = Ithaca, NY; 1 = year 1; 2 = year 2; 3 = year 3.

‡H^2, broad-sense heritability estimates, ± standard errors, on a genotype mean basis.

corresponded to increased persistence, whereas those on LG 2 corresponded to decreased persistence. In contrast, on LG 7, both parents contributed alleles associated with both increased and decreased persistence (Table 2). Each of the eight consensus linkage groups is the amalgamation of four homologs from each parent (Robins et al., 2007b). Examining the four homologs of LG 7, alleles associated with high persistence grouped together on separate homologs from alleles associated with decreased persistence (e.g., see the WISFAL-6 alleles in Fig. 1). As was previously reported for biomass yield (Robins et al., 2007b), different homologous chromosomes within the same plant can contain alleles associated with opposing effects on the trait of interest.

Interval mapping confirmed the locations of QTLs on ABI408 LG1 at Ithaca, on ABI408 LG2 for A1 and A3, and on WISFAL-6 LG7 for all 3 yr at Nashua and for A2 (Table 3; Fig. 1). These QTLs explained between 8.5 and 38.9% of the underlying phenotypic variation depending on the LG and environment (Table 3). Putative QTLs on ABI408 LG2 from A2 and on WISFAL-6 LG7 for A3 fell slightly below the 5% LOD cutoff values (both were significant at 10% LOD).

The QTLs on ABI408 LG1 resulted in lower persistence in Ithaca for genotypes receiving homologs 1 and 3 of LG 1 (Table 3). The allele ms29b2 was associated with higher persistence based on single-marker analyses (Table 2) but was located on homologs 2 and 4. On LG2, lower persistence in A1 and A3 appears to have resulted from homolog 3 because individuals with homologs 1 + 3 or 3 + 4, but not 1 + 4, showed lower persistence (Table 3). The alleles nk7 g#1av4b2, bc3c_25av2b1, and uga191b2, associated with lower persistence by single-marker analysis (Table 2), all reside on homolog 3. However, since the combination of homologs 2 + 3 did not result in lower persistence, interactions between alleles on the different homologs (including partial dominance of homolog 2 over homologs 1 and 4) may affect persistence, indicating the complications of polysomic inheritance in an autotetraploid (Rumbaugh et al., 1988) and for subsequent MAS. The QTLs on WISFAL-6 LG7 were associated with lower persistence when homologs 1 and 2 were inherited (Table 3). This reflects the negatively associated persistence alleles on homolog 2 of WISFAL-6 (aw691517a1, aw695584c1, MsaciBa1, and rc_1_51dt23v20a1) and the positively associated alleles al372288-2a1 and al373004a1 on homologs 3 and 4 (Table 2). Putative QTL positions identified by the

Table 2. Marker alleles associated with alfalfa persistence based on single marker ANOVA ($p < 0.01$) after 3 yr at Ames and Nashua, IA, and 1 yr in Ithaca, NY, with the allele effects based the presence of each specific allele.

Marker	Persistence							
	LG	A1†	A2	A3	N1	N2	N3	I
					%			
ms29b2‡	1							8§
bc3c_25av2b1	2	–6		–8				
nk7#1av4b2	2	–7	–7	–8				
uga191b2	2	–7		–7				
uga083–1a2	3							6
al372288–1a1	7	9	10	11	9	10	8	6
al373004a1	7	11§	13§	13§	10§	11	10§	
aw691517a1	7	–8	–8	–9				
aw695584c1	7	–8	–8					
bg645450b1	7	–7	–8		–7	–7	–6	
bn2_21e3v14b2	7		6					
MsaciBa1	7	–7	–8	–8				
rc_1_51dt23v20a1	7	–7	–8	–8				
rc_1_51dt23v20b2	7	6	7					
uga772b1	7	7	7		6			

†Experimental location and year: A = Ames, IA; *N* = Nashua, IA; I = Ithaca, NY; 1 = year 1; 2 = year 2; 3 = year 3.

‡Allele designations following the locus name are as follows: a = WISFAL-6, b = ABI408, c = both parents.

§Family-wise error rate significant at 10% level.

Table 3. Characterization of putative alfalfa persistence quantitative trait loci (QTLs) identified by interval mapping, including the linkage group to which each QTL belongs, the environment in which the QTL was identified, the position on the linkage group of the maximum LOD value (5% LOD thresholds as determined by 1000 permutations are in parentheses), the percentage of the phenotypic variation explained (var. exp.) by the QTL, and the phenotypic values of F_1 genotypic classes carrying specific homologous chromosomes at the QTL location.

Linkage group	Environment	QTL position	LOD value	Var. exp.	Persistence†					
					Q12	Q13	Q14	Q23	Q24	Q34
		cM		%			%			
ABI408 LG1	Ithaca	84	4.1 (3.9)	8.5	97 ± 2	87 ± 2	97 ± 2	97 ± 2	95 ± 2	96 ± 2
ABI408 LG2	Ames Year 1	78	4.8 (4.3)	14.7	95 ± 3	76 ± 3	91 ± 3	92 ± 4	94 ± 3	79 ± 3
ABI408 LG2	Ames Year 3	62	4.1 (4.1)	9.4	89 ± 3	76 ± 4	83 ± 4	86 ± 4	90 ± 4	71 ± 3
WISFAL-6 LG7	Ames Year 2	32	5.3 (5.3)	25.3	56 ± 4	95 ± 4	88 ± 3	85 ± 2	92 ± 2	95 ± 3
WISFAL-6 LG7	Nashua Year 1	32	7.3 (6.9)	33.9	50 ± 4	86 ± 3	95 ± 2	89 ± 2	92 ± 2	93 ± 2
WISFAL-6 LG7	Nashua Year 2	32	7.0 (5.6)	34.7	44 ± 4	83 ± 3	93 ± 2	87 ± 2	91 ± 2	92 ± 3
WISFAL-6 LG7	Nashua Year 3	32	8.7 (5.3)	38.9	27 ± 3	60 ± 3	68 ± 2	62 ± 2	67 ± 2	67 ± 2

†Values indicate the persistence percentages with standard errors of F1 genotypes that inherited the corresponding homologues of the respective LGs, i.e., Q12 indicates the mean persistence values of genotypes inheriting homologues 1 and 2 of the corresponding LG.

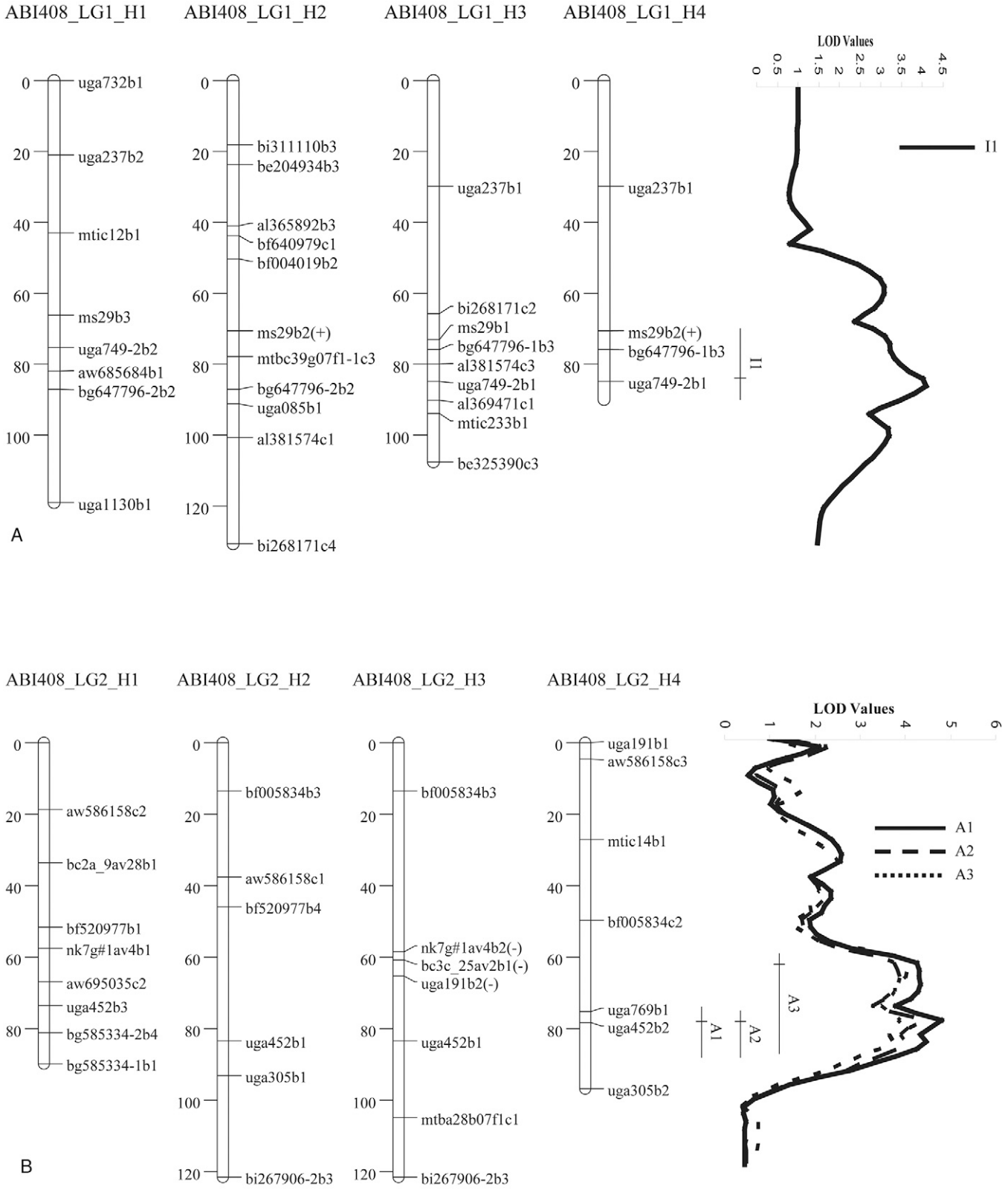

Figure 1. Location of alfalfa persistence quantitative trait loci (QTLs) based on single-marker analysis of variance and interval mapping from three locations over 3 yr on homologs of three linkage groups (A) ABI408 LG1, (B) ABI408 LG2, and (C) WISFAL-6 LG7. Location and corresponding confidence interval (LOD-1; Lander and Botstein, 1989) of QTLs identified by interval mapping are indicated to the right of the four homologs of each linkage group by a horizontal line with a tick passing through the line. The tick represents the location of the maximum LOD, and the line represents the confidence interval. Additionally, a graph of LOD values along each linkage group is included. Alleles associated with increased persistence based on single-marker analysis of variance are marked with (+) and alleles associated with decreased persistence are marked with (–). Experimental location and year: A = Ames, IA; N = Nashua, IA; I = Ithaca, NY; 1 = year 1; 2 = year 2; 3 = year 3. All putative QTLs are significant based on a 5% cutoff value with the exceptions of the A2 environment on ABI408 LG2 and the A3 environment on WISFAL-6 LG 7, which were significant based on a 10% cutoff value.

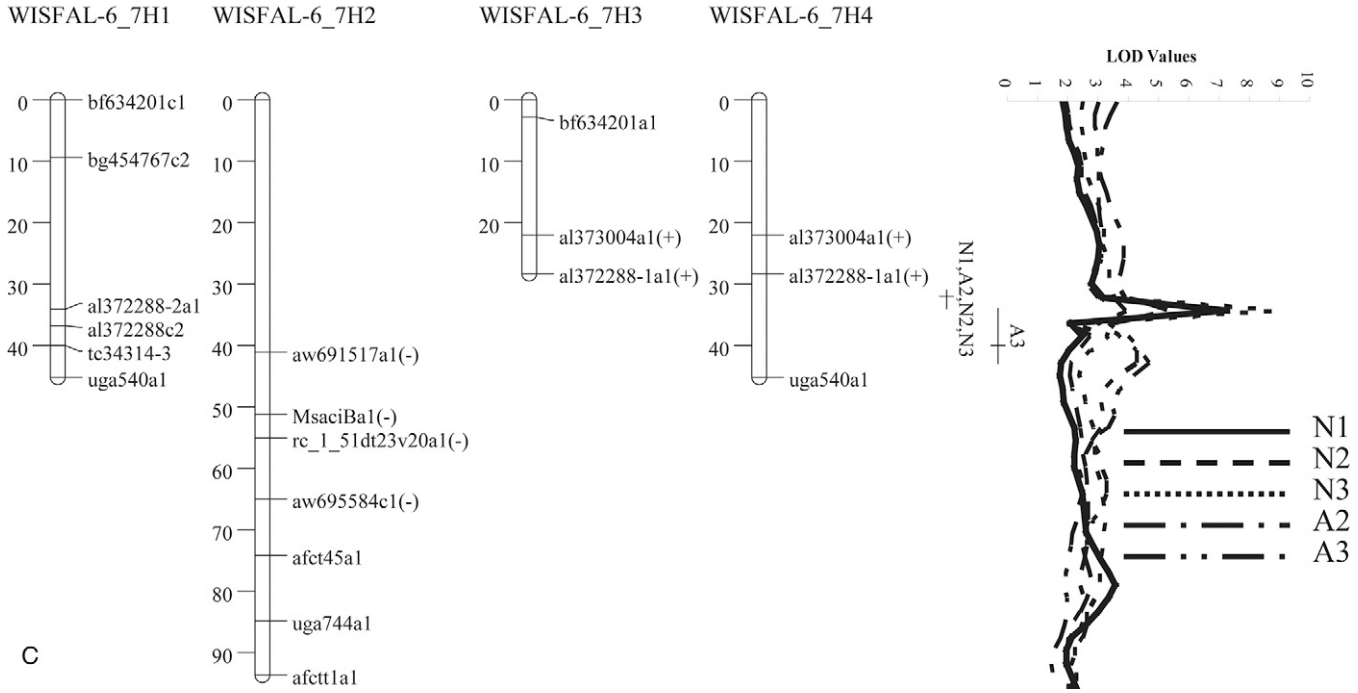

Figure 1. Continued.

single-marker analysis on WISFAL-6 LG3 and ABI408 LG7 were not identified by interval mapping.

Breeding Perspectives

We identified several QTLs for persistence in this alfalfa population. Persistence is clearly a complex trait affected by a range of biotic and abiotic stresses. Individual QTLs explained up to 39% of the phenotypic variation for persistence. Nevertheless, a large fraction of the phenotypic variation was not explained by our models, which together with the presence of at least some QTL × environment interaction indicates the complexities involved with unraveling the genetics of alfalfa persistence and complicates applications to applied breeding programs. The concordance between the two Iowa locations was stronger than either was with New York. This is not surprising and clearly shows the importance of regional breeding programs. Interestingly, the genomic regions identified as associated with persistence at a given location did not change dramatically across years. Basically, the regions identified in the first year were also identified in subsequent years, either in the QTL analysis or in the single-marker analysis, even though plant mortality increased over time, especially at Nashua. This means that location effects are stronger than year effects, and at least in this population under these conditions, markers for persistence could be identified after the first year.

Half (8 of 16) of the persistence-associated alleles also exhibited phenotypic associations with other agronomic traits, most notably, biomass production (Robins et al., 2007b). Further, the allele MsaciBa1, which produces cold-induced polypeptides, has an established link with winter hardiness (Monroy et al., 1993), so that its association with persistence is not surprising. We observed complete consistency between the biomass production results (Robins et al., 2007b) and the persistence results. In each case, alleles that were positively associated with biomass production were positively associated with persistence and vice versa. Thus, these results suggest a clear genetic connection between these traits. This may suggest that improvement in one of the traits should result in improvement in the other trait, but this is not necessarily the case. Biomass production depends on plants remaining alive, but among persistent plants, biomass can vary widely. Thus, both traits need to be evaluated simultaneously.

Further evaluation of QTLs in other populations is needed to better understand the genetic factors controlling persistence. The parents of this population are both winter hardy, and they therefore provide useful information on improving persistence from within an adapted germplasm pool. Using germplasm from different genetic backgrounds and evaluating more broadly based populations, such as is possible using association mapping, would be valuable. This experiment was conducted under space-planted conditions from rooted cuttings, which may not be applicable to sward-type production conditions (Waldron et al., 2008). These results point to potentially important breeding implications: (i) both subspecies of cultivated alfalfa have the potential to contribute alleles to increase persistence, (ii) different regions of the alfalfa genome are associated with persistence depending on the environmental conditions under which the plants are grown, highlighting the

importance of genotype by environment interaction to this trait, and (iii) a large number of the allelic associations with persistence were also identified previously as having association with other important agronomic traits, most notably biomass production (Robins et al., 2007b; Tables 2 and 3). Thus, this study was valuable in identifying preliminary regions of the alfalfa genome for further molecular characterization and potential use in marker-assisted selection.

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